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Short communication

Babesia bovis expresses a neutralization-sensitive antigen that contains a microneme adhesive repeat (MAR) domain

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ABSTRACT

A gene coding for a protein with sequence similarity to the *Toxoplasma gondii* microneme 1 (MIC1) protein that contains a copy of a domain described as a sialic acid-binding microneme adhesive repeat (MAR) was identified in the *Babesia bovis* genome. The single copy gene, located in chromosome 3, contains an open reading frame encoding a putative 181 amino acid protein, which is highly conserved among distinct *B. bovis* strains. Antibodies against both recombinant protein and synthetic peptides mimicking putative antigenic regions in the *B. bovis*-MIC1 (Bbo-MIC1) protein bind to the parasite in immunofluorescence assays and significantly inhibit erythrocyte invasion in *in vitro B. bovis* cultures. Bbo-MIC1 is recognized by antibodies in serum from *B. bovis* infected cattle, demonstrating expression and immunogenicity during infection. Overall, the results suggest that Bbo-MIC1 protein is a viable candidate for development of subunit vaccines.

Babesia bovis, a major causative agent of bovine babesiosis, is a tick-transmitted intraerythrocytic protozoan of the phylum Apicomplexa. Babesiosis is distributed throughout tropical and subtropical regions of the world [1] and is responsible for important economic losses to the livestock industry [2,3].

Although members of the phylum Apicomplexa infect different hosts and cell types, they appear to have similar host cell invasion processes [4]. Micronemal proteins designated MICs, contain neutralization-sensitive epitopes, and are key mediators of cytoadherence and invasion for *Toxoplasma gondii*. The binding properties of MIC1 to host cell receptors are due to the presence of conserved sialic acid-binding micronemal adhesive repeats (MAR) that are novel carbohydrate-binding domains found on MICs throughout the apicomplexa [5]. Each MAR domain is known to contain one central sialic acid-binding pocket [5]. Additionally, sialic acid-mediated erythrocyte invasion has been described for *B. bovis* parasites [6,7]. Thus, taking into account that a sialic acid dependent mechanism of invasion requires expression by the parasites of proteins with the ability to bind sialic acid, this study was designed to determine first whether the *B. bovis* genome codes for MAR domain containing proteins, and then whether *B. bovis* parasites express MAR containing proteins with functional relevance for erythrocyte invasion.

BLAST searches of the *B. bovis* genome [8] (http://www.ncbi.nlm.nih.gov//genomes/geblast.cgi?bact=off&gi=22172) with the *T. gondii* MIC1 protein sequence (GenBank accession no. CAA96466.1) identified a gene with significant similarity (BLAST score 8e-^{06}) on chromosome 3, with 24% identity over a stretch of 136 amino acids. This *B. bovis* gene was named *Bbo-mic1*. No additional genes with significant homology to *mic1* were found in the *B. bovis* genome (*B. bovis* T2Bo strain) using BLAST searches, indicating that this gene is likely present as a single copy. The *Bbo-mic1* gene contains an open reading frame (ORF) encoding a 181 amino acid protein with a predicted size of 21 kDa. The *Bbo-mic1* gene was amplified by PCR with primers: *Bbo-mic1* gene forward (5′-ATG GAA TTA GAC AGG ATA TGC-3′) and *Bbo-mic1* gene reverse (5′-ATG TAG ATG TAG AAA TTT AAG C-3′) from genomic DNA from *B. bovis* Mo7 [9] and R1A [10] strains from *in vitro* culture, and from *B. bovis* isolated from infected Portuguese cattle. In all cases, the PCR amplifications resulted in identical single 537 bp amplicons (data not shown), which were cloned into pCR2.1 vector (Invitrogen) and sequenced. Nucleotide sequence identity of 98% was observed among the strains (GenBank accession nos. FJ613638, FJ613636, FJ613634, FJ613635, FJ613637). Thus, the *Bbo-mic1* gene is highly conserved among these American and European strains of *B. bovis*.

Consistently, BLAST searches using the sequence of the putative Bbo-MIC1 *in silico* translated protein (GenBank accession: FJ613638) showed similarity with *T. gondii* MIC1 as well as with other predicted

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proteins from apicomplexan parasites (Fig. S1A). These related proteins differ widely in their size, and their sequence similarity is mainly restricted to an approximately 120 amino acid portions containing an arrangement of conserved cysteine residues located in the amino terminal portion of the molecules. Interestingly, a conserved domain search using the BLAST program showed that, similar to the *T. gondii* MIC1 protein, Bbo-MIC1 protein also contains a sialic acid-binding MAR motif which is located in the cysteine-rich conserved region (Fig. S1B).

Reverse transcriptase PCR (RT-PCR) amplifications of *Bbo-mic1* gene from total RNA purified from the cultured Mo7 strain was carried out by using Trizol and synthesized into cDNA using the SuperScript II RNase H reverse transcriptase (RT) first-strand synthesis system (Invitrogen, San Diego, CA, USA). The PCR reaction was performed using primers *Bbo-mic1* gene forward and *Bbo-mic1* gene reverse described above. A PCR product of 537 bp was obtained and no amplicons were produced in control PCR amplifications where the reverse transcriptase was absent (data not shown). The 537 bp amplicon was cloned into pCR 2.1 vector (Invitrogen) and then sequenced (GenBank accession no. FJ613640), confirming that the *Bbo-mic1* gene is transcribed in *in vitro* unsynchronized cultured *B. bovis* parasites.

To determine whether Bbo-MIC1 protein is expressed in *B. bovis* infected erythrocytes, we performed immunofluorescence analysis with rabbit antiserum raised against two peptides derived from the Bbo-MIC1 protein, and with pooled mouse antiserum directed against the recombinant Bbo (rBbo)-MIC1 protein. The expressed rBbo-MIC1 protein was purified from pBAD/TOPO ThioFusion (Invitrogen) clones containing a gDNA amplicon of the full size ORF of the Bbo-MIC1 gene (GenBank accession no. FJ613638) cloned in frame. The anti-rBbo-MIC1 protein mouse antiserum and both rabbit anti-peptide 1 and 2 antisera specifically recognized antigens in *B. bovis* infected erythrocytes in immunofluorescence (Fig. 1, panels A, B, C, respectively). Fluorescent parasites were patently visible around the entire microscope field. Mouse pre-immune serum (Fig. 1, panel D) and rabbit pre-immune serum (Fig. 1, panel E, F) were included as negative controls.

The reactivity of antibodies against the rBbo-MIC1 protein with *B. bovis* blood stage lysates was then tested by immunobloting. The anti-rBbo-MIC1 antibodies reacted with at least three antigens of approximately 87 kDa, 59 kDa and 21 kDa (Fig. 2A, lane 2), whereas no reactivity was observed using pre-immune mouse serum in identical immunoblots (Fig. 2A, lane 4). In addition, neither anti-rBbo-MIC1 protein-specific serum nor the pre-immune control serum reacted with normal bovine erythrocyte lysates (Fig. 2A, lanes 1 and 3, respectively). These results confirm the recognition of at least one protein with the predicted native size (~21 kDa) of the Bbo-MIC1 protein in *in vitro* cultured *B. bovis* parasites. Recognition of several antigens by the anti-rBbo-MIC1 antibodies may be interpreted as the presence of cross-reactive epitopes in other proteins, the formation of higher molecular mass multimers of the Bbo-MIC1 protein, or the occurrence of a larger precursor of the Bbo-MIC1 protein that is processed to shorter products. The latter scenario would be consistent with the presence of three closely associated groups of ORFs immediately flanking the 5′ and 3′ ends of the *Bbo-mic1* gene ORF, and separated by short intergenic regions that might result in the occurrence of a very large intron-containing gene [8]. However, mono-specific pooled polyclonal rabbit antisera raised against synthetic peptides 1 and 2 derived from Bbo-MIC1 protein that was predicted to contain B-cell epitopes and that recognizes Bbo-MIC1 protein in immunoblots, reacted only with a single antigen of approximately 21 kDa in *B. bovis* merozoite lysates (Fig. 2B, lane 1). No reactivity was observed with normal bovine erythrocyte lysates (Fig. 2B, lane 2), and no antigens were recognized by the control pre-immune rabbit serum when incubated with *B. bovis* blood stage lysates or normal bovine erythrocyte lysate (Fig. 2B, lanes 3 and 4, respectively). These findings confirm the expression of a protein with a size consistent with a sequence of the predicted Bbo-MIC1 ORF in *B. bovis* merozoites.

In addition, a serum sample from a bovine experimentally infected with the *B. bovis* T2Bo strain was tested against rBbo-MIC1 protein in immunoblots. The predicted size of the rBbo-MIC1 protein, including a ~16 thioredoxin domain at their N-terminal end and a HIS tag

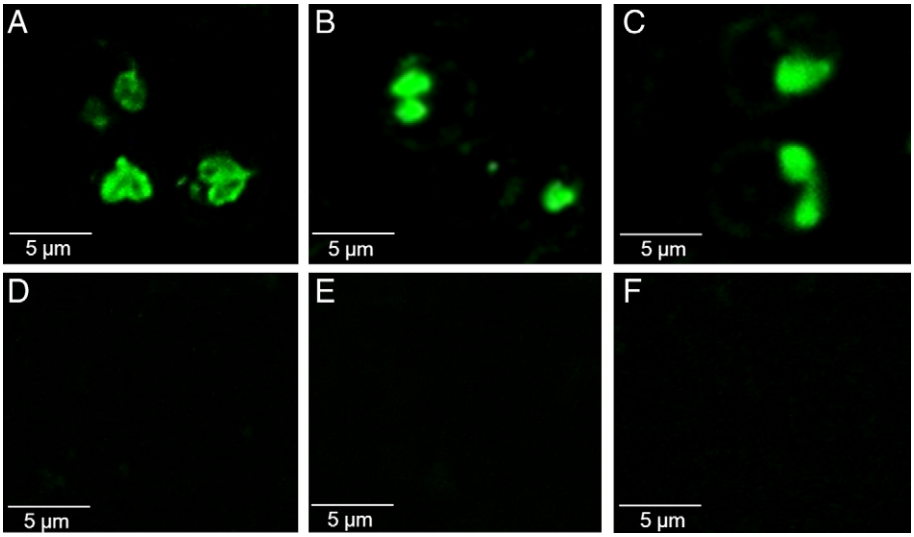

Fig. 1. Detection of the expression of the Bbo-MIC1 protein in the *Babesia bovis* infected erythrocytes (Mo7 strain) using indirect immunofluorescence assays [14]. Microscope slides made from *B. bovis* infected erythrocytes (~25% PPE) were incubated with different sera diluted 1:20 in phosphate-buffered saline (PBS) containing 1% (v/v) normal goat serum for 30 min: (A) pooled mouse antiserum against rBbo-MIC1 protein. The mouse antiserum was produced by subcutaneous inoculation of three eight-week old female BALB/c mice with 10 µg of the rBbo-MIC1 protein premixed with an equal volume of RIBI adjuvant (Corixa, Seattle, WA, USA), on days 0, 21, 42, and 63. The pool of sera used in the experiments was collected 10 days after the last booster inoculation; (B and C) rabbit antiserum against specific peptides 1 and 2 derived from the Bbo-MIC1 protein. The synthetic peptides used for rabbit immunization (Biosynthesis Company, Lewisville, TX, USA) were derived from the full-length Bbo-MIC1 protein sequence based on the presence of several charged residues and amphiphatic alpha-helices. The sequences of the synthetic peptides for antibody production used were: KEYIHEIKAATLNSLIRKNC for peptide 1 and GCYRDDEVSYDI for peptide 2 representing sequence between amino acids 62–81 and 120–131of Bbo-MIC1 respectively; (D) pre-immune mouse serum; (E and F) pre-immune rabbit serum. The slides probed with mouse sera were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) G, (KPL) and the slides probed with rabbit sera were incubated with FITC-conjugated goat anti-rabbit IgG (KPL). Epifluorescence was examined at 630× with a Nikon Confocal microscope.

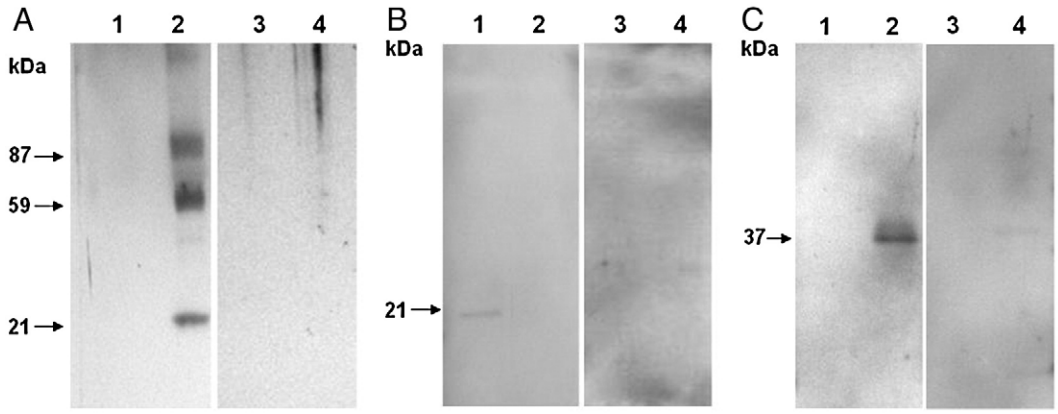

Fig. 2. Panel A: immunoblot analysis of lysates of non-infected bovine erythrocytes (lanes 1 and 3) and *Babesia bovis* Mo7-infected bovine erythrocytes (lanes 2 and 4), using different sera diluted 1:200 in PBS containing 0.2% Tween 20 (PBST). Lanes 1 and 2 show reactivity of pooled immune mouse sera against rBbo-MIC1 protein, and lanes 3 and 4 show reactivity of pre-immune mouse. Individual bands and their corresponding molecular masses are indicated by arrows. Primary antibodies were incubated for 40 min and the binding reaction was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (DAKO Carpinteria, CA, USA). Panel B: immunoblot analysis of lysates of *B. bovis* Mo7-infected bovine erythrocytes (lanes 1 and 3) and lysates of non-infected bovine erythrocytes (lanes 2 and 4), using sera diluted 1:200 in PBST buffer containing 5% skim milk. Pooled rabbit antisera against specific peptides 1 and 2 derived from the Bbo-MIC1 protein (lanes 1 and 2) or pre-immune rabbit sera (lanes 3 and 4) was incubated with the membranes as described above. The immunoblots were developed using an HRP-conjugated goat anti-rabbit IgG (DAKO). Panel C: recognition of rBbo-MIC1 protein by serum from *B. bovis* infected bovine in immunoblots. Lanes 1 and 3: control unrelated recombinant *A. marginale* protein; lanes 2 and 4: purified rBbo-MIC1 protein. Lanes 1 and 2 were incubated with serum from a bovine experimentally infected with the *B. bovis* Mo7 strain, and lanes 3 and 4 were incubated with serum from an uninfected bovine. All cattle sera used in the blots were previously diluted 1:20 in PBST containing 0.2% I-block. A band of approximately 37 kDa recognized only by the experimentally infected bovine serum is indicated by an arrow. The immunoblots were developed using a HRP-conjugated goat anti-bovine IgG (KPL, Gaithersburg, Maryland, USA). All immunoblots shown in Fig. 2 were incubated for 30 min with their respective conjugated-antibody previously diluted 1:10,000 in PBST, and developed using a chemiluminescence ECL Plus kit (GE Healthcare, Pittsburgh, PA, USA) in an X-ray film.

domain at its C-terminal end is of ~37 kDa. Consistently, the immune serum tested recognized a ~37 kDa antigen when incubated with the purified rBbo-MIC1 protein in immunoblots (Fig. 2C, lane 2). No reactivity was observed with either an unrelated *B. bovis* protein, an *A. marginale* protein (Fig. 2B, lane 1) that was produced in the same way as rBbo-MIC1 or with control serum from an uninfected bovine (Fig. 2B, lanes 3 and 4, respectively). We also analyzed the pattern of reactivity of sera from four additional cattle experimentally infected with the T2Bo strain of *B. bovis* and sera from five uninfected cattle by immunobloting. The four sera obtained from experimentally infected cattle also recognized the ~37 kDa rBbo-MIC1 protein, whereas no reactivity was observed with any of five normal cattle sera (data not shown). These results indicate that Bbo-MIC1 protein is expressed during *B. bovis* infection in cattle and that it contains B-cell epitopes recognized by the immune system of the bovine during the course of the infection.

To test whether the Bbo-MIC1 protein, similar to related apicomplexan MIC1 proteins, is involved in erythrocyte invasion by *B. bovis* merozoites, an *in vitro* neutralization assay was performed according to Hines et al. [11]. Briefly, free merozoites were pre-incubated with the following pooled sera: polyclonal rabbit antiserum against peptides 1 and 2 of Bbo-MIC1 protein, pre-immune rabbit serum, polyclonal mouse antiserum against rBbo-MIC1 protein, and control pre-immune mouse serum. In addition, the previously characterized neutralization-sensitive monoclonal antibody BABB35A4 which recognizes *B. bovis* MSA-1 [11,12], and polyclonal control mouse antiserum directed against a non-*Babesia* related recombinant protein (*A. marginale* ORF2) were used as positive and negative controls, respectively, in *in vitro* neutralization assays.

A statistically significant lower percentage of parasitized erythrocytes (PPE) (95.7% ± 0.5% inhibition) ($P<0.005$) was measured 72 h after the initiation of the cultures when polyclonal mouse antiserum against rBbo-MIC1 protein was added to the *in vitro* cultures (Table 1). In contrast, neither pre-immune serum nor control antiserum (Table 1) had a significant effect on the PPEs, ($P>0.05$). In addition, pooled polyclonal rabbit antiserum against peptides 1 and 2 of Bbo-MIC1 protein were also able to significantly inhibit erythrocyte invasion by the parasites (45.1% ± 6.8% inhibition) ($P<0.005$) (Table 1). These results suggest that the Bbo-MIC1 protein contains neutralization-sensitive B-cell and is surface exposed, thus it might be targeted by the immune system of the bovines.

In summary, we have identified a neutralization-sensitive molecule expressed by *B. bovis* with similarity to the *T. gondii* MIC1 protein, including the conservation of a sialic acid-binding MAR domain [5]. It is remarkable that similar to *Plasmodium* parasites [13], *B. bovis* uses sialic acid-binding domains to invade erythrocytes, while in contrast, *T. gondii* is able to invade multiple cell targets, with the exception of erythrocytes [6,7]. The presence of *in vitro* neutralization-sensitive B-cell epitopes in the BboMIC1 protein suggests that this protein has functional relevance in host cell recognition and invasion by *B. bovis*. The collective data suggest that the Bbo-MIC1 protein could be included as an additional

Table 1
In vitro inhibition of parasite invasion using antibodies against the Bbo-MIC1 protein [9]. Percentage of parasitized erythrocytes (PPE) was calculated daily in Giemsa-stained blood smears for 3 days after the incubation of the *B. bovis* Mo7 cultures parasites with different sera. All sera were previously heat-inactivated for 30 min at 56 °C, diluted 1:1 in culture medium and incubated with free merozoites for 30 min at 4 °C. After the incubation, an equal volume of 5% v/v bovine erythrocytes in culture medium was added to each well and the 96-well plate was incubated at 37 °C in a 5% CO_2. The percentages of inhibition of erythrocyte invasion were calculated using the PPE obtained 72 h after the start of the cultures.

Treatment	Inhibition of parasite invasion (%)[a]
None	0
Rabbit pre-immune serum	12.9(±4.6)
Rabbit immune serum against Bbo-MIC1 specific peptides 1 and 2 [b]	45.1(±6.8)*
Mouse pre-immune serum	18.5(±4)
Mouse immune serum against rBbo-MIC1 protein[c]	95.7(±0.5)*
Monoclonal antibody BABB35A4 (anti *B. bovis* MSA-1)[d]	69.4(±3.2)*
Mouse serum anti-*A. marginale* ORF2	3(±5.9)

An asterisk (*) indicates $P<0.005$. Results from three independent experiments and using triplicate wells were analyzed by the one-tailed Student's *t*-test.

[a] Percentages of invasion (±SD) were calculated using the following formula: 100−(PPE in experimental culture well × 100)/PPE in control culture well.

[b] Pool of the rabbit immune sera against Bbo-MIC1 specific peptide previously designated as 1 and 2.

[c] Pool of mice immune serum against rBbo-MIC1 protein.

[d] The neutralization-sensitive monoclonal antibody BABB35A4 is reactive with *B. bovis* MSA-1 [9].

target for the development of new methods for controlling *B. bovis* infection in cattle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.parint.2010.03.004.

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